

**Export systems for recombinant proteins****Description**

5 The present invention relates to vectors, host-  
vector combinations and processes for preparing stable  
fusion proteins consisting of a carrier protein and a  
passenger protein, where expression of the fusion  
proteins leads to exposure of the passenger domains on  
10 the surface of bacterial cells, especially Escherichia  
coli cells. If required, the passenger domains can be  
released into the medium by proteases, for example by  
selected host factors such as, for example, OmpT.

15 The present invention further relates to the  
use of carrier proteins or carrier protein portions  
from natural proteins which are present as amino-acid  
sequences in data banks or files and are called, in  
accordance with their properties, autotransporters.

20 Methods for identifying and selecting bacteria  
which express at least one passenger protein on their  
surface with defined affinity for a binding partner,  
and the use thereof for diagnostic purposes, are made  
possible by the present invention. In particular, the  
process according to the invention allows peptide  
libraries to be expressed on the surface of bacterial  
25 cells, with the aid of which it is possible, for  
example, to determine the ligands having the highest  
affinity in the case of antibodies, MHC molecules or  
other components of the immune system.

30 Also made possible by the process according to  
the invention is the production of fusion proteins  
which are composed of portions of heavy and light  
antibody domains and an autotransporter, and transport  
thereof through the bacterial cell coat. In a specific  
embodiment, finally, the targeted variation of  
35 recombinant antibodies with binding activity, and their  
functional presentation on the cell surface of  
Escherichia coli become possible.

The process according to the invention  
generally allows recombinant proteins, which may be

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receptors or ligands, to be expressed on the bacterial surface, and selection on the basis of the binding affinity for a binding partner, which makes selection, associated therewith, of a clonal producer possible.

5           The use of bacteria which express protein fusions on the cell surface and which are present bound to a carrier material or in solution for the specific enrichment or purification of a binding partner showing affinity for protein domains exposed on this surface is  
10 also according to the invention. Furthermore, the present invention also relates to the surface expression of enzymes or other proteins with biologically, chemically or industrially relevant properties, and, where needed, the specific release  
15 thereof into the surrounding medium.

          The exposure of recombinant proteins on the surface of bacterial cells is a method with a large number of possible microbiological, molecular biological, immunological or industrial applications.  
20 Production of recombinant proteins in this manner makes their properties, for example binding affinities or enzymatic activities (Francisco et al., Bio. Technology 11 (1993) 491-495) available without a further step such as, for example, disruption of the producer cell  
25 being necessary. Since only a limited number of factors are naturally expressed on the bacterial surface, there is in addition specific enrichment of the recombinant protein by comparison with cytosolic production. Another considerable advantage is that the same methods  
30 used to select the recombinant protein which is sought can also be used to isolate the producer of this protein, a bacterial cell, and thus a clonal producer which can be permanently stored, stably reproduced and grown on a large scale can be obtained.

35           Various systems have been used to date for the presentation of recombinant proteins on the cell surface, but these without exception are also used naturally for the transport or secretion of bacterial surface proteins (Little et al., TIBTECH 11 (1993),

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3-5). Significantly, in these cases the DNA region which naturally codes for the protein to be transported, the passenger, was replaced or supplemented by the coding DNA region of the required recombinant protein, although the coding regions of the protein domains responsible for the transport, the carrier proteins, usually remained unchanged. It is clear from this that systems in which passenger and carrier components are present immediately adjacent or encoded in one gene, so-called one-component systems, have a considerable advantage by comparison with systems having several independent components (Gentschev et al., Behring Inst. Mitt. 95 (1994) 57-66), especially in the production of universally usable vectors which, besides the property of stable replication, one or more selection markers, and the protein domains needed for transport, must also contain an insertion site for the DNA fragment encoding the passenger. The carrier proteins used in many one-component systems used to date have been E. coli outer membrane proteins. These include, inter alia, LamB (Charbit et al., Gene 70 (1988), 181-189), PhoE (Agterberg et al., Gene 59 (1987), 145-150) or OmpA (Francisco et al., Proc. Natl. Acad. Sci (1992), 2713-2717), whose use entails disadvantages, however. Thus, additional protein sequences can be integrated only in loops exposed on the surface, which on the one hand leads to fixed amino- and carboxyl-terminal ends on the flanking carrier protein sequences, and on the other hand has a limiting effect on the length of the sequences to be introduced. Although the use of peptidoglycan-associated lipoprotein (PAL) as carrier protein leads to transport to the outer membrane, no presentation of native protein sequences on the surface of E. coli is possible therewith (Fuchs et al., Biol. Technology 9 (1991), 1369-1372). Surface expression of relatively large proteins is possible using a fusion of OmpA and Lpp as carrier protein portion, to whose carboxyl end the passenger protein sequences are

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attached (Franscisco et al., Proc. Natl. Acad. Sci (1992), 2713-2717). A disadvantage which has to be accepted in this case is that the fixing of the N-terminus of the passenger may prevent correct folding or functioning.

Also known are so-called autotransporter-containing proteins, a family of secreted proteins in Gram-negative bacteria. The publication of Jose et al. (Mol. Microbiol. 18 (1995), 377-382) mentions some examples of such autotransporter proteins. These proteins contain a protein domain which enables an N-terminally attached protein domain to be transported through a pore structure formed from  $\beta$ -pleated sheet structures in the outer membrane of Gram-negative bacteria. The autotransporter-containing proteins are synthesized as so-called polyprotein precursor molecule. The typical structure of such a precursor protein is divided into three. At the N-terminus there is a signal sequence which is responsible for the transport through the inner membrane, taking advantage of the Sec transport apparatus present in the host and being deleted during this. To this is attached the protein domain to be secreted, followed by a C-terminal helper domain which forms a pore in the outer membrane, through which the N-terminally attached protein domain to be secreted is translocated to the surface. Depending on its function to be carried out, the latter remains there linked to the helper, which is now serving as membrane anchor, on the bacterial surface, or is deleted by proteolytic activity, and this proteolytic activity may be intrinsic to the protein domain to be secreted or be a property derived from the host or be an external/specifically added activity (for example thrombin, IgA protease). Secretion of heterologous polypeptides or proteins using an expression system based on an autotransporter is known. Thus, for example, it is known from EP-A-0 254 090 or the publication of Klauser et al. (EMBO J. 11 (1992), 2327-2335) that the helper domain of the IgA protease

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from *N. gonorrhoeae* can express heterologous polypeptides as passenger domains in the heterologous bacterial strains *E. coli* and *Salmonella typhimurium*.

In addition, the extracellular transport of the protein VirG by shigella is described in Suzuki et al. (J. Biol. Chem. 170 (1995) 30874-30880). This protein is likewise an IgA protease-like autotransporter which is capable of the expression of foreign polypeptides such as, for example, MalE and PhoA, which have been covalently linked to the N terminus of the autotransporter domain of VirG. In addition, the paper by Shimada et al. (J. Biochem, 116 (1994), 327-334) describes the extracellular transport of a heterologous polypeptide, namely pseudoazurin from *A. faecales*, in *E. coli* using the autotransporter domain of the serine protease from *S. marcescens*.

In the processes described in the prior art for preparing for the expression of heterologous passenger proteins with the aid of autotransporter systems, however, considerable disadvantages have been found. Thus, on use of the transporter or helper domain of the IgA protease from *N. gonorrhoeae* in *E. coli* as host strain, considerable compatibility problems frequently arise. Excessive expression leads to cytolysis or the bacteria show reduced growth even with moderate expression, which in both cases leads to a considerable reduction in the yield of fusion protein and points to weaknesses in the stability of the system. The present invention was thus based on the technical problem of providing carrier proteins which, especially on use of *E. coli* as host strain, do not lead to these disadvantages because, for a variety of reasons, *E. coli* is to be preferred to, for example, *Neisseria gonorrhoeae* as host strain. On the one hand, *E. coli* strains with recombinant DNA can be cultured even in simple laboratories of safety level 1. In addition, *E. coli* strains have already been used in the commercial production of recombinant proteins. This means that there is a considerable advantage in the

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handling and manipulation of recombinant E. coli strains by comparison with other host strains. In addition, a large number of accurately characterized mutant strains of E. coli already exist and permit a selection of the host strain depending on the required use.

This problem is solved by a method for presenting peptides or/and polypeptides on the surface of Gram-negative host bacteria, where

- 10 a) there is provision of a host bacterium which is transformed with a vector on which is located, operatively linked to a promoter, a fused nucleic acid sequence comprising:
  - 15 (i) a signal peptide-encoding nucleic acid section,
  - (ii) a nucleic acid section coding for the passenger peptide or/and passenger polypeptide to be presented,
  - (iii) where appropriate a nucleic acid section coding for a protease recognition site,
  - 20 (iv) a nucleic acid section coding for a transmembrane linker and
  - (v) a nucleic acid section coding for a transporter domain of an autotransporter;
  - 25 and
- (b) the host bacterium is cultivated under conditions with which there is expression of the fused nucleic acid sequence and presentation of the peptide or polypeptide encoded by the nucleic acid section (ii) on the surface of the host bacterium, characterized in that the nucleic acid section (ii) is heterologous relative to the nucleic acid section coding for the transporter domain (v), and the host bacterium is homologous relative to the nucleic acid section coding for the transporter domain (v).
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- 35

It is surprisingly possible by using a host bacterium which is homologous relative to the nucleic acid section coding for the transporter domain to achieve a surface presentation of peptides or and

polypeptides, in particular including short synthetic peptides with a length of, preferably, 4-50 amino acids or of eukaryotic polypeptides, which is distinctly improved by comparison with the prior art.

5           In the process according to the invention there is provision of a host bacterium which is transformed with one or with a plurality of compatible recombinant vectors. A vector of this type contains, operatively linked to a promoter and, where appropriate, other  
10 sequences necessary for expression, a fused nucleic acid sequence. This fused nucleic acid sequence comprises (i) a signal peptide-encoding section, preferably a section which codes for a Gram-negative signal peptide which makes passage through the inner  
15 membrane into the periplasm possible. The fused nucleic acid sequence (ii) also comprises a section coding for the passenger peptide or polypeptide to be presented. A nucleic acid section coding for a protease recognition site is, where appropriate, located on the 3' side of  
20 this section (iii). Examples of suitable protease recognition sites are recognition sites for intrinsic, that is to say naturally present in the host cell, or externally added proteases. Examples of externally added proteases are the IgA protease (compare, for  
25 example, EP-A-0 254 090), thrombin or factor X. Examples of intrinsic proteases are OmpT, OmpK or protease X. On the 3' side of this section there is located (iv) a nucleic acid section coding for a transmembrane linker, which makes presentation of the  
30 peptide or polypeptide encoded by section (iii) on the outside of the outer membrane of the host bacterium possible. On the 3' side of this section is a nucleic acid section coding for a transporter domain of an autotransporter.

35           The transmembrane linker domains particularly preferably used are homologous in relation to the autotransporter, that is to say the transmembrane linker domains are encoded by nucleic acid sections directly on the 5' side of the autotransporter domains.

The length of the transmembrane linkers is preferably 30-16 amino acids.

5 The transporter domain is able to form a so-called  $\beta$ -barrel in the outer membrane of the host bacterium. The  $\beta$ -barrel consists of an even number of antiparallel, amphipatic,  $\beta$ -pleated sheets. This structure has, like other proteins of the outer membrane of Gram-negative bacteria, an aromatic amino acid such as phenylalanine or tryptophan at the  
10 C terminus. This is followed alternately by charged (polar) and uncharged (hydrophobic) amino acids, and this structure appears to play a part in the folding with the membrane. The number and location of the amphipatic,  $\beta$ -pleated sheets can be identified with the  
15 aid of a suitable computer program and be used, with the aid of analogies to the outer membrane porins whose crystal structure is known (Cowan et al., Nature 358 (1992) 727-733), for constructing a model of the barrel structure. The barrel structure is preferably  
20 constructed as follows: 9-14, in particular about 12 amino acids (AA) for a membrane passage; no or a minimal number of charged AA point outwards in a  $\beta$ -pleated sheet; small loops, or none at all, point inwards, where appropriate large or very large loops  
25 point outwards; the  $\beta$ -barrel is composed of 12, 14, 16 or 18, in particular 14, antiparallel  $\beta$ -pleated sheets.

Starting from the model of the barrel, it is now possible for the region necessary for self-transport through the outer membrane to be established  
30 and linked by a signal peptide and a passenger domain at the genetic level. Expression of this construct then makes transport of the passenger protein to the bacterial surface possible, it being possible for the signal peptide to derive originally from the passenger  
35 or from another protein. It must be taken into account in this connection that a linker region which is of suitable length and sequence and which extends through the pore which has formed and ensures that the

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passenger domains are completely exposed on the surface is also linked properly to the  $\beta$ -barrel.

An essential feature of the process according to the invention is that the host bacterium is homologous relative to the nucleic acid section coding for the transporter domain, that is to say the host cell and the transporter domain are selected from homologous families, for example enterobacteria, preferably from homologous genera, for example escherichia, salmonella, or helicobacter, particularly preferably from homologous species, for example Escherichia coli, Salmonella typhimurium. It is particularly preferred to use salmonella or E. coli as host bacterium and a transporter domain which is likewise derived from salmonella or E. coli, or a variant thereof.

A particularly suitable E. coli host strain which may be mentioned here is the strain JK321 (DSM 8860) which is ompT<sup>-</sup>, dsbA<sup>-</sup> and carries the genetic marker fpt, which leads to stable surface expression even of large proteins such as, for example, the V<sub>H</sub> chain of an antibody with the aid of the iga $\beta$  helper protein.

In a preferred embodiment, the present invention therefore relates to a carrier protein which performs an autotransporter function and makes surface exposure of recombinant proteins possible in Escherichia coli with high yield. In a typical example, this is the autotransporter of the "adhesin involved in diffuse adherence" (AIDA-I) from E. coli (Benz and Schmidt, Infect. Immun. 57 (1989), 1506-1511). The transporter domain of the AIDA-I protein is depicted in Fig. 2. Besides this specific sequence, it is also possible to use variants thereof which can be produced, for example, by modifying the amino-acid sequence in the loop structures not involved in the membrane passage. It is also possible, where appropriate, for the nucleic acid sections coding for the surface loops to be completely deleted.

It is also possible within the amphipathic  $\beta$ -pleated sheet structures to carry out conservative amino acid exchanges, that is to say replacement of one hydrophilic by another hydrophilic amino acid or/and replacement of one hydrophobic by another hydrophobic amino acid. A variant preferably has a homology of at least 80% and, in particular, at least 90% with the sequence, indicated in Fig. 2, of the AIDA-I autotransporter domain, at least in the region of the  $\beta$ -pleated sheet structures.

In another typical example, the autotransporter used is that of the SepA protein from *Shigella flexneri* (Benjellou-Touimi et al., Mol. Microbiol 17 (1995) 123-135) or a variant thereof. In another typical example, it is the autotransporter of the IcsA protein from *Shigella flexneri* (Goldberg et al., J. Bacteriol 175 (1993), 2189-2196) or of the Tsh protein from *E. coli* (Provence et al., Infect. Immun 62 (1994), 1369-1380). In another typical example, it is the autotransporter of the Hsr protein from *Helicobacter mustelae* (O'Toole et al., Mol. Microbiol. 11 (1994), 349-361), of the Prn protein from *Bordetella* ssp. (Charles et al., Proc. Natl. Acad. Sci USA 86 (1989), 3554-3558; Li et al., J. Gen. Microbiol. 138 (1992), 1697-1705), of the Ssp protein from *Serratia marcescens* (for example in Yanagida et al., J. Bacteriol. 166 (1986), 937-944 or Genbank Accession No. X59719, D78380), of the Hap protein from *Haemophilus influenzae* (StGeme et al., Mol. Microbiol. 14 (1994), 217-233), of the BrkA protein from *Bordetella pertussis* (Fernandez and Weiss, Infect. Immunol. 62 (1994), 4727-4738), of the VacA protein from *Helicobacter pylori* (Schmitt and Haas, Mol. Microbiol. 12 (1994), 307-319) or various rickettsial proteins (for example 190kDa cell surface antigens, Genbank Accession No. M31227; SpaP, Carl et al., Proc. Natl. Acad. Sci. USA 87 (1990), 8237-8241; rOmpB, Gilmore et al., Mol. Microbiol. 5 (1991), 2361-2370 and Slp T, Hahn et al., Gene 133 (1993), 129-133) or a variant thereof as defined above.

The DNA sequences, and the amino-acid sequences derived therefrom, of the aforementioned auto-transporters are depicted in Figures 7-24.

Further autotransporter domains in bacterial surface proteins or in secreted bacterial proteins may be derived from protein sequences present in data banks from in protein sequences which are based on DNA sequences available in data banks, or from protein sequences determined by sequence analysis directly or indirectly via the DNA sequence. The corresponding coding regions (genes) can be used to prepare vectors or fusion protein genes which make efficient surface expression of passenger proteins possible in Gram-negative bacteria, especially *E. coli*.

Surface presentation or exposure means according to the invention that the fusion proteins or passenger domains are located on the side of the outer bacterial membrane facing the medium. In intact Gram-negative bacteria, passenger proteins exposed on the surface are freely accessible to binding partners.

In a preferred embodiment, the present invention thus makes possible the surface presentation of peptides or, in another embodiment, the surface presentation of peptide libraries in Gram-negative bacteria, especially in *E. coli*, and the use thereof for determining the affinity for an antibody or another receptor or for epitope mapping. Epitope mapping means that the peptide with the greatest affinity for an antibody or another receptor is identified exposed on the surface of the producing strain. This makes clear a crucial advantage of the present invention by comparison with previously used phage systems (Makowski, Gene 128, (1953), 5-11) for expressing peptide libraries. In the bacterial system according to the invention, identification of a peptide having the required properties takes place simultaneously with the selection of the clonal producer. The latter can be grown directly and used to produce larger amounts of the required peptide without the need for the elaborate

cycles of infection (phage replication) and selection (phage selection) as with the phage system. The growing of the strain expressing the correct peptide exposed on the surface takes place over the same time as  
5 amplification of the corresponding coding gene, sequence analysis of which permits unambiguous identification and characterization of the peptide with simple and established techniques. These advantages according to the invention apply to all passenger  
10 domains expressed exposed on the surface using the present invention, that is to say peptides and polypeptides.

A peptide library produced according to the invention thus contains fusion proteins composed of an  
15 autotransporter, in a particularly preferred embodiment of the AIDA autotransporter, and of a peptide which is produced, exposed on the surface, in a Gram-negative bacterium, preferably *E. coli*. The wide variety of different expressed peptides results in a typical  
20 example from the cloning of degenerate, synthetically prepared oligonucleotides between the coding regions for the signal peptide and the autotransporter.

In another preferred embodiment, the present invention makes it possible to express proteins or  
25 protein fragments acting as antigen on the surface of Gram-negative bacteria, preferably *E. coli*. The construction of a fusion protein of this type takes place according to the invention using the  $\beta$  subunit of the toxin *Vibrio cholerae* (CtxB) as passenger and the  
30 AIDA autotransporter as carrier protein. The accessibility of the surface-exposed antigenic domains for suitable binding partners has been demonstrated according to the invention by labelling with an antiserum specific for CtxB. It emerged from this that  
35 the recombinant fusion proteins embedded in the outer membrane of the *E. coli* host strain may comprise up to 5% of the total cell protein, which means a considerably improved efficiency by comparison with other systems. The process described here thus makes

possible the stable production and presentation of proteins or protein fragments having antigenic activity on the surface of Gram-negative bacteria and, in a preferred embodiment, the use thereof as live vaccine, for oral vaccination or for screening sera or antibody banks. The use of bacterial cells, for example attenuated salmonella strains (Schorr et al., Vaccine 9 (1991) 675-681) with proteins which have antigenic activity and are expressed exposed on the surface has proved advantageous in live vaccination by comparison with the intracellular bacterial expression of antigens.

The present invention generally permits, in a preferred embodiment, the surface expression of all passengers which are in their essential constituent peptides or proteins on the surface of Gram-negative bacteria, in particular E. coli.

In another preferred embodiment, the C-terminal domain of the AIDA protein, the AIDA autotransporter, serves as membrane anchor for the presentation of recombinant polypeptides of the immune system, for example recombinant antibody domains on the surface of Gram-negative bacteria. Surface expression of recombinant antibody fragments makes it possible to modify them rapidly and to assess and investigate their antigen-binding properties. Thus, it becomes possible to produce whole libraries of functional antibody fragments exposed on the surface, and to test them for particular given binding properties or affinities. The advantage of the present invention by comparison with previously used phage systems is that the variation, that is to say the genetic manipulation and the production of the protein, can take place in the same host organism. It is moreover possible for the genetic manipulation to be targeted (site-specific mutagenesis) or random, using degenerate oligonucleotides to synthesize an intact fusion of antibody-encoding fragment as passenger and the autotransporter as carrier protein. It is likewise possible for the

genetic manipulation to take place in the form of in vivo mutagenesis by exposing the bacteria which contain the gene for the fusion protein to high-energy radiation (for example UV) or chemical agents having  
5 mutagenic effects.

The selection, according to the invention, of the molecule having the correct binding properties takes place alongside the selection of the producing bacterial cell. It is evident from this that this  
10 procedure according to the invention, in its strategy consisting of variation and subsequent selection, is based on the natural strategy of the immune system for the best possible adaptation of binding properties of immunogenic molecules. Various procedures according to  
15 the invention are conceivable for expressing functional antigen-binding parts of antibodies, which are not usually glycosylated, on the surface of Gram-negative bacteria, preferably E. coli. Two monovalent fragments can be presented together through separate fusions of  
20 the light chain (VL) and the heavy chain (VH) with, in each case, an autotransporter domain, which are expressed independently of one another with different compatible vectors or under the control of different promoters on the same vector in a host cell. The two  
25 antibody domains which are present exposed on the surface assemble to form a functional Fv fragment on the surface, it being possible for the stability of the interaction to be promoted by chemically induced disulphite bridge formation or another type of chemical  
30 crosslinking.

In another procedure according to the invention there is preparation of fusion proteins which contain the autotransporter as carrier protein, and as passenger the light chain (VL) and the heavy chain (VH)  
35 of an antigen-binding domain of an antibody, linked via a short linker peptide (for example [Gly<sub>4</sub>Ser]<sub>3</sub>) which permits correct assembly of the two chains to form a functional Fv fragment. For construction of such single-chain (sc) Fv fragments, it is possible both to

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link the N terminus of the light chain to the C terminus of the heavy chain, and to link the N terminus of the heavy chain to the C terminus of the light chain (Pluckthun Immun. Rev. 130 (1992), 151-188). It is also possible using the procedures described to produce a complete Fab fragment.

In another preferred embodiment, the present invention makes possible the surface-exposed expression of MHC class II molecules in *E. coli*, where appropriate with defined embedded peptides. Two strategies are conceivable for this. In one variant, two different fusion proteins, both of which contain an autotransporter as carrier protein, are expressed on separate compatible vectors or on one vector under the control of different promoters in a host cell. The passenger protein employed is, on the one hand, the  $\alpha$  chain of the required MHC class II subtype and, on the other hand, the  $\beta$  chain of this subtype, to whose N terminus the required peptide can be attached via a linker (Kozono et al., Nature 369 (1994) 151-154).

In the second variant, a passenger protein consisting of the peptide, the  $\beta$  chain and the  $\alpha$  chain is fused to an autotransporter. The  $\alpha$  chain and  $\beta$  chain assemble on the bacterial surface to form a functional MHC molecule, with the peptide being correctly embedded in the binding cavity. The stability of the complex can be assisted by a chemically induced disulphide bridge formation. Variation of the embedded peptide is possible by site-specific mutagenesis or/and by using degenerate oligonucleotide primers in the preparation of the DNA fragments encoding the fusion proteins, as well as by in vivo mutagenesis methods using high-energy radiation or/and chemical mutagens.

Once again, the advantage of the process according to the invention becomes clear. Variation of the binding partner, expression, selection of the molecule having the optimal properties, sequence analysis and stable production can take place in one host strain. This also makes it possible, for example

for variants of previously known ligands with improved binding properties to be rapidly characterized, and thus optimization of ligands or receptors.

5 In another preferred embodiment, the present invention makes possible the surface expression of immunomodulatory receptors such as, for example, CD1, Fc receptor or MHC class I molecules, and specific variation thereof.

10 In another preferred embodiment, the present invention makes possible the surface expression of T-cell receptors or parts thereof, but also of other surface antigens of eukaryotic cells or cells of the immune system.

15 In another preferred embodiment, the protein fragments or peptides expressed on the surface are T-cell epitopes which, following uptake of the bacteria by appropriate cell lines or primary cells such as, for example, macrophages, presented as peptides embedded in MHC molecules of class I or II and can serve to  
20 stimulate specific T cells.

In a particularly preferred embodiment, the process according to the invention makes possible the surface expression and the variation of a peptide or polypeptide having an affinity for a binding partner,  
25 of a ligand, of a receptor, of an antigen, of a toxin-binding protein, of a protein having enzymatic activity, of a nucleic acid-binding protein, of an inhibitor, of a protein having chelator properties, of an antibody or of an antigen-binding domain of an  
30 antibody.

The term "binding partner" means according to the invention an element, a molecule, a chemical compound or a macromolecule, where the binding partner and/or the bacterial cells expressing the fusion  
35 proteins are in a freely soluble form, bound to a matrix or else associated with a biological membrane.

The term "antigen-binding domain" refers according to the invention to at least the region of an

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antibody molecule which is sufficient for specific binding of an antigen.

In another preferred embodiment, the present invention makes chemical, physical or/and enzymatic modification of the passenger peptide or polypeptide, or parts thereof, exposed on the surface possible, it being possible for the modification to be a covalent modification, a non-covalent modification, a glycosilation, a phosphorylation or a proteolysis.

The process according to the invention for producing a variant population of peptides exposed on the surface and for identifying bacteria which carry peptides or polypeptides having a particular required property is divided into the following steps:

- (1) preparation of one or more fusion genes by cloning the coding sequence of a required passenger in frame with the coding sequence of a transporter domain of an autotransporter and of a signal peptide, it being possible for the individual subfragments to be amplified by PCR or to derive from restriction digestions of other DNA, in at least one vector;
- (2) variation of the passenger by mutagenesis, for example by site-specific mutagenesis, using degenerate oligonucleotide primers in the PCR, by chemical mutagenesis or by using high-energy radiation;
- (3) introduction of the vector or vectors into host bacteria;
- (4) expression of the fusion gene or fusion genes in the host bacteria which present the fusion protein or fusion proteins stably on the surface;
- (5) cultivation of the bacteria, for example in liquid culture or on agar plates, to produce the

passenger presented stably exposed on the surface  
or the passengers presented stably exposed on the  
surface;

- 5 (6) where appropriate selection of the bacteria which  
carry the passenger or passengers having the  
required properties on the surface, and
- 10 (7) where appropriate characterization of a binding  
partner for the passenger having the optimal  
properties.

It is moreover possible according to the  
invention to perform this process several times in  
15 order to adapt the properties of the surface-exposed  
peptide or polypeptide stepwise to the required binding  
behaviour, or to optimize, in a first step, the binding  
partner in respect of one property and, in a second  
step, in respect of one or more other properties.

20 However, it is also possible according to the  
invention, depending on the required use, to link only  
a few constituent steps of the process together, in a  
typical example the constituent steps (1), (3), (4) and  
(5), but also all other possible combinations.

25 In a preferred embodiment of this process, the  
fusion protein contains as carrier protein the  
autotransporter domain of the AIDA protein or a variant  
thereof which makes secretion of the fusion protein  
possible.

30 In another preferred embodiment of this  
process, the fusion protein contains as carrier protein  
the SepA autotransporter or a part thereof, or the IcsA  
autotransporter or a part thereof, or the Tsh  
autotransporter or a part thereof, or the Ssp  
35 autotransporter or a part thereof, or the Hap  
autotransporter or a part thereof, or the Prn  
autotransporter or a part thereof, or the Hsr  
autotransporter or a part thereof, or the BrkA  
autotransporter or a part thereof, or the VacA

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autotransporter or a part thereof or a rickettsial autotransporter or a part thereof, each of which makes secretion of the fusion protein possible.

5 The expression of multimeric proteins is possible according to the invention by preparing in one cell different fusion proteins which assemble on the surface to form a functional unit.

10 The short generation time of the bacteria used as host organism makes it possible to have a permanent variation and selection cycle which makes it possible to adapt, in an evolutionary manner, the passenger protein, but also the autotransporter, to given properties. This may involve, in a typical example, the binding affinities between the passenger protein and a  
15 binding partner. The isolation of the bacteria having the stably exposed fusion protein takes place, in a preferred embodiment of this process, by binding to an immobilized or/and labelled binding partner, for example a matrix-fixed binding partner, to a binding  
20 partner with a fluorescent label, a binding partner labelled with magnetic particles, or a binding partner with a chromogenic label. In another preferred embodiment, the binding partner is modified so that it can be detected in a second step by a binding partner  
25 specific for the modification.

Another aim of the present invention is to provide stably expressed fusion proteins or parts thereof or fusion proteins expressed stably on the surface of bacteria, and the use thereof for  
30 therapeutic purposes or diagnostic purposes, in pollutant concentration or removal, in the inactivation of toxins, in the mobilization of raw materials, in food production or processing, in detergent production, in the labelling of selected eukaryotic or prokaryotic  
35 cells. It is possible according to the invention to use bacteria expressing antibodies or antibody fragments stably on the surface, in a typical example using the AIDA autotransporter as transporter domain, for the production thereof, these antibodies or antibody

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fragments subsequently being employed, where appropriate after purification, for diagnostic or therapeutic purposes. It would be possible, for example, to use such antibodies or antibody fragments to identify or select specifically cells with particular surface markers, a typical example which may be mentioned here being tumour antigens. In another typical example, the labelled surface markers are receptors, in which case the labelling takes place along with the blocking of the or one of the receptor properties, which makes it possible specifically to inhibit a signal transduction induced or mediated by the receptor, and the cell function associated therewith.

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#### **Description of the figures**

Figure 1:

Hydrophobicity of the C-terminal 300 amino acids of the AIDA-I protein.

The pore typical of autotransporters in the outer membrane of Gram-negative bacteria is formed by amphipatic  $\beta$ -pleated sheet structures, that is to say by domains with  $\beta$ -pleated sheet structure and alternating hydrophobic and hydrophilic amino acids. This can be demonstrated by plotting a relative hydrophobicity value of the amino acid, which has been assigned to the amino acid by means of a particular algorithm, against the position of the amino acid. The algorithm of Vogel and Jähnig (J. Mol Biol. 190 (1986) 191-199) was used. The arrows show the possible membrane passages, with an arrow to the left denoting that the membrane passage runs from the inside to the outside and an arrow to the right indicating a membrane passage from the outside to the inside. SP indicates a relative surface probability of the amino acids calculated by the method of Emini et al. (J. Virol. 55 (1985), 836-839).

Figure 2:

Model of the autotransporter from the AIDA-I protein.

Starting from the plot of the relative hydrophobicity of an amino acid against its position (Figure 1), the barrel structure formed by the antiparallel, amphipatic  $\beta$ -pleated sheets can be depicted as model. The barrel structure which is depicted here cut open is closed in the membrane by interaction of the first with the antiparallel last membrane passage. The amino acids written inside rhombi are located in the membrane region, with those surrounded by thick lines being relatively hydrophobic and being oriented towards the outside of the barrel, that is to say towards the membrane, while those surrounded by thin lines are relatively hydrophilic and point with their side chains towards the inside of the pore. Amino acids shown in circles form loops outside the membrane. Alanine at position 1 of the model has the number 1014 in the complete sequence of the AIDA-I, while the terminal phenylalanine has the number 1286 in the complete sequence (Benz and Schmidt, Mol Microbiol 11 (1992), 1539-1546).

Figure 3a:

Preparation of pJM7, a vector for surface expression of CtxB.

pJM7 contains a gene fusion (FP59) of cholera toxin B and the AIDA linker/ $\beta$ -barrel region. This gene fusion is expressed constitutively under the control of the artificial promoter PTK (Klauser et al., EMBO J. 9 (1990) 1991-1999) in a vector with high copy number. The ctxB gene was amplified by PCR using the oligonucleotides EF16 and JM6 from the plasmid pTK1 (Klauser et al. EMBO J. 9 (1990) 1991-1999). The autotransporter consisting of the  $\beta$ -barrel and the linker region from AIDA-I was amplified by amplification using the oligonucleotides JM1 and JM7 from a plasmid DNA preparation from E. coli EPEC 2787 (Benz and Schmidt, Infect. Immun. 57 (1989),

1506-1511). The oligonucleotide JM1 contains in its 5' projection a BglII recognition sequence, and oligonucleotides JM6 and JM7 each contain a KpnI recognition sequence. The vector DNA (pBA) was hydrolysed with ClaI and BamHI, and the two PCR products were then, following the amplification, cut with ClaI and KpnI (EF16/JM6 fragment) or with BglII and KpnI (JM7/JM1 fragment). The three fragments generated in this way were condensed in a ligation.

Figure 3b:

Preparation of pJM22, a vector for surface expression of peptides.

pJM22 produces the fusion protein FP50 which consists of three domains. At the N-terminal end there is located the CtxB signal sequence which ensures export of the resulting fusion protein through the cell membrane (Sec mediator). This is followed by the passenger domain, in this case a peptide, the epitope PEYFK. At the C-terminal end of the fusion protein is the AIDA  $\beta$ -barrel/linker region, the autotransporter, which conveys the passenger domain with N-terminal truncation by the signal peptide to the surface of E. coli. To construct pJM22, firstly the DNA of pJM7 was hydrolysed with XhoI, and the vector portion of pJM7 was amplified by PCR using the oligonucleotides JM7 and JM20. This entailed deletion of the ctxB gene apart from its signal sequence. The oligonucleotide JM20 contained in its 5' overhang, in addition to the KpnI cleavage sequence, five codons which code for the amino acids PEYFK. This amino-acid sequence represents a linear epitope for the monoclonal antibody D $\mu$ 142. The PCR product was hydrolysed with KpnI and then self-ligated.

Figure 4

Expression detection and protease sensitivity

Because of the strong stable expression of the fusion proteins FP59 (derived from pJM7) and FP50

(derived from pJM22) in *E. coli*, these can easily be identified in a whole cell lysate stained with Coomassie brilliant blue. Protease accessibility represents a conventional means for determining the location of a protein. Access is to be expected to cell-intrinsic proteins only if these are presented on the outside of the bacterium or if the outer membrane of the bacterium is permeable to proteases. To rule out the latter, it is possible to use a protease-sensitive marker which is known to be naturally present in the periplasm. The integrity of the outer membrane is ensured only if this marker is not attacked by the protease employed. Cells of *E. coli* UT5600 or JK321 were cultured overnight on LB agar (50 mg/l ampicillin) and suspended in PBS. The cell suspensions were adjusted to an OD<sub>578</sub> = 4.0. Cells from 0.5 ml of cell suspension were sedimented for 1 min in a bench centrifuge and resuspended in 200 µl of PBS with 0.1 mg/ml protease. The mixtures were incubated at 37°C for 20 min and stopped by cooling to 0°C, sedimenting for 1 minute and resuspending the pellet in 40 µl of SDS-PAGE sample buffer and immediately boiling for 15 minutes. The evaluation took place after SDS-PAGE by Western blotting (4b and 4c) or by staining with Coomassie brilliant blue (4a). Access of the proteases to the periplasm was ruled out by employing not only antisera specific for the passenger protein domains but also an antiserum specific for the C-terminal part of OmpA, which is naturally present inaccessibly in the periplasm and ought therefore not be capable of being attacked by externally added proteases such as trypsin (4c).

Figure 4a:

SDS-PAGE and subsequent staining with Coomassie brilliant blue to detect protease sensitivity and quantify expression. Whole cell lysates of *E. coli* JK321 and *E. coli* UT5600 were loaded.

Lane 1 JK321 pJM7 C \*

Lane 2 JK321 pJM7 T\*\*

Lane 3 JK321 pJM7 -\*\*\*

Lane 4 Molecular weight markers (94, 67, 43, 30, 20  
5 and 14 kDa)

Lane 5 JK321 pJM22 C

Lane 6 JK321 pJM22 T

Lane 7 JK321 pJM22 -

Lane 8 JK321 pTK61 C

10 Lane 9 JK321 pTK61 T

Lane 10 JK321 pTK61 -

Lane 11 UT5600 pJM7 C

Lane 12 UT5600 pJM7 T

Lane 13 UT5600 pJM7 -

15 Lane 14 Molecular weight markers (94, 67, 43, 30, 20  
and 14 kDa)

Lane 15 UT5600 pJM22 C

Lane 16 UT5600 pJM22 T

Lane 17 UT5600 pJM22 -

20 Lane 18 UT5600 pTK61 C

Lane 19 UT5600 pTK61 T

Lane 20 UT5600 pTK61 -

C\* Cells were digested with chymotrypsin

25 T\*\* Cells were digested with trypsin

\*\*\* Native cells

Figure 4b:

Western blot for detecting expression and protease  
30 sensitivity

Whole cell lysates of E. coli JK321 and E. coli  
UT5600 were loaded. After the electrophoresis, the  
proteins were transferred from the gel by the semi-dry  
method to a nitrocellulose membrane. The filters were  
35 then blocked with blocking solution (PBS with 0.5%  
Tween 20 and 0.5 M NaCl) for 10 min, and the first  
antiserum, AK55 (rabbit anti-cholera toxin B) diluted  
1:200 in blocking solution, was added. To detect the  
epitope PEYFK, the hybridoma supernatant Dül42, diluted



1:35 in blocking solution, was added. The filters were incubated with the primary antibodies for 1 h, then washed three times and incubated with protein A-alkaline phosphatase conjugate (1:500 in blocking solution) for 30 min. The filters were developed with NBT/BCIP colour solution.

	Lane 1	JK321 pJM7 C *
	Lane 2	JK321 pJM7 T**
10	Lane 3	JK321 pJM7 -***
	Lane 4	Molecular weight markers (106, 80, 50, 32, 27 and 18 kDa)
	Lane 5	JK321 pJM22 C
	Lane 6	JK321 pJM22 T
15	Lane 7	JK321 pJM22 -
	Lane 8	JK321 pTK61 C
	Lane 9	JK321 pTK61 T
	Lane 10	JK321 pTK61 -
	Lane 11	UT5600 pJM7 C
20	Lane 12	UT5600 pJM7 T
	Lane 13	UT5600 pJM7 -
	Lane 14	Molecular weight markers (106, 80, 50, 32, 27 and 18 kDa)
	Lane 15	UT5600 pJM22 C
25	Lane 16	UT5600 pJM22 T
	Lane 17	UT5600 pJM22 -
	Lane 18	UT5600 pTK61 C
	Lane 19	UT5600 pTK61 T
	Lane 20	UT5600 pTK61 -

30

C\* Cells were digested with chymotrypsin

T\*\* Cells were digested with trypsin

-\*\*\* Native cells

35

Figure 4 c:

Demonstration of the integrity of the outer membrane by Western blot analysis.

Whole cell lysates of E. coli JK321 and E. coli UT5600 were loaded. After the electrophoresis, the

proteins were transferred from the gel by the semi-dry method to a nitrocellulose membrane. The filters were then blocked with blocking solution (PBS with 0.5% Tween 20 and 0.5 M NaCl) for 10 min, and the first antiserum, K56 (rabbit anti-OmpA) diluted 1:1000 in blocking solution, was added. The filters were incubated with the primary antibodies for 1 h, then washed three times and incubated with protein A-alkaline phosphatase conjugate (1:500 in blocking solution) for 30 min. The filters were developed with NBT/BCIP colour solution. OmpA is an outer membrane protein of *E. coli* with a C-terminal periplasmic portion. This periplasmic part is trypsin-sensitive. If trypsin has access to the periplasm, a part about 10-11 kDa in size is digested off mature OmpA (35 kDa). Digestion would thus result in a displacement of the OmpA band in the Western blot from 35 kDa to 25 kDa (Klauser et al., EMBO J. 9 (1990) 1991-1999), which is obviously not the case on use of the AIDA-I autotransporter for transporting recombinant proteins.

	Lane 1	JK321 pTK1 T*
	Lane 2	JK321 pJM7 T
	Lane 3	JK321 pJM22 T
25	Lane 4	JK321 pTK61 T
	Lane 5	Molecular weight markers (106, 80, 50, 32, 27 and 18 kDa)
	Lane 6	JK321 pTK1 -**
	Lane 7	JK321 pJM7 -
30	Lane 8	JK321 pJM22 -
	Lane 9	JK321 pTK61 -
	Lane 10	empty
	Lane 11	UT5600 pTK1 T*
	Lane 12	UT5600 pJM7 T
35	Lane 13	UT5600 pJM22 T
	Lane 14	UT5600 pTK61 T
	Lane 15	Molecular weight markers (106, 80, 50, 32, 27 and 18 kDa)
	Lane 16	UT5600 pTK1 -**

Lane 17 UT5600 pJM7 -  
Lane 18 UT5600 pJM22 -  
Lane 19 UT5600 pTK61 -

- 5 T\* Cells were digested with trypsin  
\*\* Native cells

Figure 5  
Immunofluorescence

10 Immunofluorescence of whole, non-permeabilized  
cells represents a conventional method for detecting  
determinants exposed on the cell surface. Antibodies  
employed therein for detecting the determinants are too  
large to pass through the intact outer membrane. The  
15 control used for differentiation and for estimation of  
the background activity of periplasmically or  
cellularly expressed determinants comprises antibodies  
against antigens known to be expressed periplasmically  
or cellularly respectively.

20 Cells of E. coli UT5600 which contain one of  
the plasmids pBA, pTK1, pTK61, pJM7 or pJM22 were  
cultured overnight on LB agar (ampicillin 50 mg/l) and  
suspended in PBS to an optical density of 0.1 at  
578 nm. 500 µl of this cell suspension were used to  
25 coat cover glasses which were placed in 24-well  
microtitre plates. The cells were sedimented onto the  
cover glasses in a plate centrifuge for 5 min. 450 µl  
of the supernatant were aspirated off and replaced by  
PBS with 2.5% PFA (paraformaldehyde), with which  
30 fixation was carried out for 20 min. The supernatant  
was completely aspirated off and three washes with  
500 µl of PBS were carried out. Nonspecific binding  
sites were blocked by incubation with 300 µl of PBS  
containing 1% FCS for 5 min. The blocking solution was  
35 completely aspirated off, and the cover glasses were  
centred in their wells, covered with 15 µl of a 1:100  
dilution of the rabbit serum AK55 (raised against  
cholera toxin B) and incubated in a humidity chamber at  
room temperature for 1 h. This was followed by three

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washes with 500  $\mu$ l of PBS each time, blocking with 350  $\mu$ l of PBS/FCS for 5 min, and incubation with 15  $\mu$ l of 1:100 dilution of a goat anti-rabbit-Texas red conjugate for 30 min. After a subsequent three washes, the cover glasses were placed on slides and embedded using embedding medium. The result of the immunofluorescence was assessed under the microscope and recorded by photography with exposure times of equal length.

- 10 a) E. coli UT5600 pBA (strain used as negative control containing only the cloning vector without insert)
- 15 b) E. coli UT5600 pTK1 (produces cholera toxin B which is exported into the periplasm. This construct is used for determining the background activity of the periplasmically expressed cholera toxin B).
- 20 c) E. coli UT5600 pJM7 (expresses FP59, the fusion protein of AIDA and cholera toxin B, which is presented on the surface of E. coli).
- 25 d) E. coli UT5600 pJM22 (expresses FP50, the fusion protein of AIDA and the epitope PEYFK. This construct is used to demonstrate that the AIDA portion of FP59 and FP50 shows no cross-reactivity with the AK55 used in this experiment).
- 30 e) E. coli UT5600 pTK61 (produces a fusion protein of cholera toxin B and Iga- $\beta$  which is presented on the surface of E. coli (Klauser et al., EMBO J. 9 (1990) 1991-1999). Used for comparison with the AIDA construct FP59).

#### Figure 6

DNA sequences of the oligonucleotides used

#### 35 Figures 7-24

DNA sequence (non-coding strand) and amino-acid sequences derived therefrom, of bacterial autotransporters.

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Figure 7

Depiction of the membrane-integrated part of the AIDA-I autotransporter from *Escherichia coli* (Benz and Schmidt, Mol. Microbiol. 6 (1992), 1539-1546).

5

Figure 8

Depiction of the membrane-integrated part of the BrkA autotransporter from *Bordetella pertussis* (Fernandez and Weiss, Infect. Immun. 62 (1994), 4727-4738).

10

Figure 9

Depiction of the membrane-integrated part of the Hap autotransporter from *Haemophilus influenzae* (StGeme et al., Mol. Microbiol. 14 (1994), 217-233).

15

Figure 10

Depiction of the membrane-integrated part of the Hsr autotransporter from *Helicobacter mustelae* (O'Toole et al., Mol. Microbiol. 11 (1994), 349-361).

20

Figure 11

Depiction of the membrane-integrated part of the IcsA autotransporter from *Shigella flexneri* (Goldberg et al., J. Bacteriol. 175 (1993), 2189-2196).

25

Figure 12

Depiction of the membrane-integrated part of the Prn (outer membrane protein P96) autotransporter from *Bordetella pertussis* (Charles et al., Proc. Natl. Acad. Sci. USA 86 (1989), 3554-3558).

30

Figure 13

Depiction of the membrane-integrated part of the Prn (P70 pertactin) autotransporter from *Bordetella parapertussis* (Li et al., J. Gen. Microbiol. 138 (1992), 1697-1705).

35

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Figure 14

Depiction of the membrane-integrated part of the 190 kDA cell surface antigen autotransporter from *Rickettsia rickettsii* (Anderson et al., unpublished, Genbank Accession No. M31227).

Figure 15

Depiction of the membrane-integrated part of the SpaP autotransporter from *Rickettsia prowazekii* (Carl et al., Proc. Natl. Acad. Sci. USA 87 (1990), 8237-8241).

Figure 16

Depiction of the membrane-integrated part of the 120 kilodalton outer membrane protein (rOmp B) auto- transporter from *Rickettsia rickettsii* (Gilmore et al., Mol. Microbiol. 5 (1991), 2361-2370).

Figure 17

Depiction of the membrane-integrated part of the SlpT autotransporter from *Rickettsia typhi* (Hahn et al., Gene 133 (1993), 129-133),

Figure 18

Depiction of the membrane-integrated part of the SepA autotransporter from *Shigella flexneri* (Benjellou-Touimi et al., Mol. Microbiol. 17 (1995), 123-135).

Figure 19

Depiction of the membrane-integrated part of the Ssp autotransporter from *Serratia marcescens* RH1 (Rho, unpublished, Genbank Accession No. X59719).

Figure 20

Depiction of the membrane-integrated part of the Ssp autotransporter from *S. marcescens* IFO-3046, clone pSP11 (Yanagida et al., J. Bacteriol. 166 (1986), 937-944).

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Figure 21

Depiction of the membrane-integrated part of the Ssp-h1 autotransporter from *Serratia marcescens*, strain IFO3046 (Onishi and Horinouchi, unpublished, Genbank Accession No. D78380).

Figure 22

Depiction of the membrane-integrated part of the Ssp-h2 autotransporter from *Serratia marcescens*, strain IFO3046 (Onishi and Horinouchi, unpublished, Genbank Accession No. D78380).

Figure 23

Depiction of the membrane-integrated part of the Tsh autotransporter from *Escherichia coli* (Provence et al., 1994, Infect. Immun. 62 (1994), 1369-1380).

Figure 24

Depiction of the membrane-integrated part of the VacA autotransporter from *Helicobacter pylori* (Schmitt and Haas, Mol. Microbiol. 12 (1994), 307-319). At least 3 other forms of VacA are also known in *Helicobacter pylori*, but they differ in the stated region to an inconsiderable extent.

EXAMPLES

Example 1:

Identification and localization of the autotransporter in a surface protein of *Escherichia coli*.

In order to find an autotransporter appropriate for the required use, that is to say adapted to the passenger protein and the host strain to be used, it is necessary to carry out an analysis of the C-terminal amino-acid sequence of a protein under consideration. This may be a protein already identified as surface factor, or else an amino-acid sequence, deposited in a data bank, of a protein of unknown function, or an amino-acid sequence, derived from a DNA sequence

deposited in a data bank, of a protein, or the amino-acid sequence, derived from a gene following a sequence analysis, of a protein. The N terminus of the protein ought to contain a signal peptide sequence in order to  
5 make transport across the inner membrane possible, and the part integrated into the membrane ought to start at the C terminus with the aromatic amino acid phenylalanine or tryptophan, followed by alternately polar (or charged) and hydrophobic (or aromatic) amino  
10 acids. The passenger domain ought to contain few cysteines and no disulphide bridges at all, since it has emerged that this blocks transport of the passenger through the pore which is formed. The hydrophobicity plot ought to indicate an even number of amphipatic  
15  $\beta$ -pleated sheet structures from which the outer membrane pore is constituted. The amphipatic  $\beta$ -pleated sheet structures ought to be about about 12 amino acids long and contain a minimum amount of charged amino acids oriented towards the membrane side, with the  
20 loops joining the membrane passages containing few amino acids towards the periplasm. Considerably more amino acids can be present towards the outside (medium). The results of this in the hydrophobicity plot are an assembly of the membrane passages in  
25 antiparallel pairs with the exception of the first and the last membrane passage, which complete the barrel structure of the pore by assembling together in antiparallel fashion. Based on compliance with these criteria, it is now possible to construct a model of  
30 the autotransporter, which can be used to establish the location and extent of the amino acids necessary for the transport. In addition to the amino acids needed for the pore, the fusion protein must also include, for an autotransporter capable of functioning, a so-called  
35 linker region which runs from the N terminus, located in the periplasm, of the  $\beta$ -barrel structure through the pore to the surface, so that the surface exposure of all the passenger domains is completely ensured.

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The first aim of the present invention was to provide a system for optimized surface exposure of recombinant proteins in E.coli. This is why an autotransporter was sought in a natural surface protein of E.coli. The choice fell on the adhesin AIDA-I (Adhesin Involved in Diffuse Adherence, Benz and Schmidt Infect. Immun. 57 (1989) 1506-1511), whose sequence was available in data banks. A signal sequence of 49 amino acids at the N terminus was shown according to the invention, while the requirements according to the invention at the C terminus were met by the amino-acid sequence FSYKI (phenylalanine-serine-tyrosin-lysine-isoleucine). The transported domain contained no cysteines, and the hydrophobicity plot (Figure 1) predicted 14 antiparallel, amphipatic  $\beta$ -pleated sheet structures. Thus, to form the pore, at least the amino acids from alanine at position 1014 of the complete amino-acid sequence (Benz and Schmidt, Mol. Microbiol. 6 (1992) 1539-1546) up to phenylalanine at position 1286 are necessary (Figure 2). Additionally selected as linker region were amino acids attached to alanine 1014 on the N-terminal side. The functional autotransporter region selected in this way could then be isolated by PCR from the DNA of the corresponding E.coli EPEC2787 and used to construct a fusion protein.

Example 2:

Construction of a surface-exposed fusion protein having an antigenic determinant as passenger protein

Based on the assumptions that AIDA-I is an autotransporter and that a gene fusion of any desired passenger and an autotransporter intrinsic to E.coli (namely AIDA- $\beta$ ) ought to be more compatible with E.coli than a gene fusion of the same passenger with a heterologous autotransporter (for example Iga- $\beta$ ), a gene fusion was produced between aida- $\beta$  and a gene for a passenger protein. In order to ensure transport of the passenger, not only AIDA- $\beta$  but also a connecting

region ("linker") located on the N-terminal side of the  $\beta$ -barrel was cloned.

CtxB was selected as passenger, and the corresponding gene from pTK1 (Klauser et al, EMBO J. 9 (1990), 1991-1999) was amplified by PCR using the oligonucleotides EF16 and JM6. Since AIDA-I is plasmid-encoded in E.coli EPEC 2787 (Benz and Schmidt, Infect. Immun. 57 (1989), 1506-1511), the AIDA-I autotransporter with linker region was likewise amplified from a plasmid preparation of E.coli EPEC 2787 by PCR using the oligonucleotides JM1 and JM7. The two PCR products were digested with restriction enzymes whose recognition sequences were present in the oligonucleotides. The two fragments produced in this way were cloned into an appropriately predigested cloning vector (pBA) with high copy number. This resulted in a construct with an artificial constitutive promoter (PTK; Klauser et al., EMBO J. 9 (1990) 1991-1999) in front of a gene fusion consisting of ctxB at the 5' end (coding for amino acids 1-113), followed by an AIDA-I linker (coding for amino acids 116-279 of the fusion protein) and the AIDA-I autotransporter (coding for amino acids 280-563 of the fusion protein) at the 3' end (Figure 3a). The resulting gene fusion was called FP59.

The expression, which was substantially greater than with the previously existing Iga- $\beta$  system and which was achieved without the tendency to lysis which is to be observed with Iga- $\beta$ , was unambiguously demonstrated by comparative electrophoresis of whole cell lysates (Figure 4a).

Demonstration of the surface exposure of FP59 was provided by various methods. The protease sensitivity of FP59 was shown in the protein gel by a reduction in the molecular weight following addition of trypsin or chymotrypsin (Figure 3a). Protease-resistant fragments with, in each case, a mass of about 33-35 kDa were produced (Figure 3a). These protease-resistant fragments contain no immunogenic portions of the

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passenger protein. This was shown by Western blot analysis of whole cell lysates using an anti-cholera toxin B serum and comparing with protease-digested and undigested FP59-expressing E.coli (Figure 4b and  
5 comparison of 4a and 4b).

Partial N-terminal sequencing of the membrane-protected trypsin-digested products revealed that the membrane linker region in the AIDA autotransporter has a length of 55 amino acids.

10 It was also possible with the protease digestions to show the integrity of the outer membrane of FP59-expressing E.coli (Fig. 4c). For this purpose, whole cell lysates were, following the trypsin digestion, developed by immunoblotting with an anti-  
15 OmpA serum. Both undigested cells and trypsin-digested cells showed intact OmpA as was to be expected for cells with an intact outer membrane.

It was also possible to show surface exposure and strong expression of FP59 by immunofluorescence studies (Figure 5). It is possible by binding  
20 fluorescence-labelled antibodies to demonstrate the surface exposure of an antigen on a bacterial cell with an intact outer membrane. This was shown by FP59-expressing E.coli cells by strong fluorescence. The  
25 E.coli cells used as negative controls, with periplasmically expressed cholera toxin B, with surface-exposed FP50 (Figure 3b) and with non-recombinant cloning vector were unambiguously negative. The  
periplasmic cholera toxin B demonstrated the  
30 inaccessibility of the periplasm for antibodies (Figure 5b), and the negative result of the immunofluorescence with FP50 made it possible to rule out cross-reactivity of the antiserum used (against the  
passenger protein) with the AIDA portions of FP59  
35 (Figure 5d). The immunofluorescence with the non-recombinant cloning vector was a measure of the background staining intrinsic to the method of measurement (Figure 5a). This also made it possible to compare the expression of FP59 with B61, the surface-

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presented cholera toxin B-Iga- $\beta$  fusion protein produced by pTK61 (Figures 5c and 5e), likewise making it possible to demonstrate an unambiguous advantage of the novel system according to the invention.

5

Example 3:

Construction of a surface-presented peptide fusion

10 A peptide which acts as linear epitope for a monoclonal antibody (Dü142) was presented and detected on the surface. The peptide was cloned using a PCR-dependent strategy which is extremely suitable for the generation and surface exposure of peptide libraries. This entails formation of a triple gene fusion of the  
15 export signal of ctxB (bases 1-81), of a short sequence coding for a peptide (bases 82-96) and of the aida linker/aida- $\beta$  region (bases 103-1450).

pJM7 (Figure 3a) was linearized with XhoI and used as template (Figure 3b) for a PCR with the  
20 oligonucleotides JM7 and JM20 (Figure 6). Both oligonucleotides had a KpnI recognition sequence at their 5' ends [lacuna] JM7 was chosen so that, on use thereof in a PCR, the aida linker/aida- $\beta$  domains were amplified. JM20 was chosen so that the PCR product  
25 contained the signal sequence present in ctxB for the Sec-dependent membrane transport through the cytoplasmic membrane and the six codons subsequent thereto. In addition, JM20 contained in its 5' extension, which was not complementary to the template,  
30 five codons which coded for the linear epitope of the antibody Dü142. The KpnI recognition sequence was located upstream of these codons. After the PCR, the resulting product was hydrolysed with KpnI, self-ligated and then transformed into E.coli. Correct gene  
35 fusions were identified by colony immunoblotting (no figure). Expression and surface exposure were demonstrated in analogy to the methods described in Example 2 by Western blot analysis of protease digests

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and analysis of protein stainings in the gel (Figures 4a, b, c).

5 The generation of extensive peptide libraries  
can be done by slightly modifying the cloning strategy  
described herein. The division described for JM20 of  
the various functional regions of this oligonucleotide  
must for this purpose be altered so that the region  
coding for the linear epitope is replaced by a region  
10 which is deliberately subjected to degeneration during  
the oligonucleotide synthesis. Degeneration means that,  
in place of defined bases at all position of this  
functional region, there is replacement of single,  
multiple or all bases by a base mixture composed of up  
15 to four different bases. This means that each codon can  
code for up to 20 different amino acids, instead of for  
one amino acid, resulting in a pool of coding sequences  
which are theoretically possible for all conceivable  
combinations of amino acids in a peptide of the given  
length. The cell which carries the peptide having the  
20 required property can now be isolated, mediated by  
binding of the surface-exposed peptide to a binding  
partner, which, for example, is in a form immobilized  
on a matrix, has a fluorescent label or is coupled to  
magnetic beads, and be used for continual production  
25 and characterization.

add  
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